Limitations of HPLC for the Detection of β-Exotoxin in Culture Filtrates of *Bacillus thuringiensis*

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Summary. High performance liquid chromatography (HPLC) was used to detect and quantify β-exotoxin, a phosphorylated adenine nucleotide derivative produced as an excreted metabolite by several strains of *Bacillus thuringiensis*. The assay was rapid and quantitative for purified β-exotoxin standards. However, peak height failed to correlate β-exotoxin concentration in crude culture filtrates with biological activity toward house fly larvae. Unrelated compounds (from non-β-exotoxin producers) co-eluted with β-exotoxin, thereby making the technique an unreliable method for toxin detection and quantification.

Introduction

In 1959, McConnell and Richards reported the discovery of a heat-stable, dialyzable toxin produced by certain strains of the insect pathogen *Bacillus thuringiensis*. The production, toxicity, chemistry, and mode of action of this toxin have been reviewed by several authors (Bond et al. 1971; Vankova 1978). The thermostable toxin, also known as β-exotoxin or thuringiensin, is easily distinguishable by host spectrum and structure from the proteinaceous inclusion body (δ-endotoxin) also produced by *B. thuringiensis*. The preferred assay method for β-exotoxin employs house fly larvae *(Musca domestica)*, which fail to develop into normal adults following exposure to the toxin (Mechalas and Beyer 1963; Cantwell et al. 1964). This bioassay suffers from several limitations, including inaccurate potency estimations of impure toxin and poor reproducibility.

Alternatives to the house fly bioassay are few. One alternative is a microbiological assay which involves the growth inhibition of *Sarcina flava* (Rosenberg et al. 1971). A second choice is an isotope dilution method for the estimation of radioactively-labeled β-exotoxin in culture (Sebesta et al. 1973). Because neither method has been routinely adopted for quantification of β-exotoxin, the laborious and inconsistent house fly bioassay has remained the method of choice for β-exotoxin measurement.

Recently, however, Oehler et al. (1982) recommended the use of high-performance liquid chromatography (HPLC) as an alternate method for β-exotoxin measurement. The authors cited rapid and direct measurement of β-exotoxin in crude culture filtrates as well as in purified samples. In this study, we have investigated the application of HPLC for β-exotoxin detection and quantification.

Materials and Methods

Organisms and Media. Strains of *B. thuringiensis* were grown on a modified nutrient sporulation medium with phosphate (NSMP) (Johnson et al. 1975) or yeast extract-glucose (YEG) (Yousten and Rogoff 1969) at 30° C with constant agitation. To stimulate β-exotoxin production, sucrose (2–10%) was substituted for glucose (Shieh and Rogoff 1973). Depending upon experimental requirements, cultures were incubated from 8–60 h. Culture broths were centrifuged and filter sterilized (Millipore 0.45 micron membrane), and the filtrates were assayed without further treatment or were heated (80° C for 10 min) and concentrated in vacuo prior to assay.

HPLC. High performance liquid chromatographic separations were made by the method of Oehler et al. (1982) with a Waters Associates M-6000 pump, a C_{18} μBondapak reverse-phase column (4 mm × 25 cm), and a Model 77 ultraviolet (UV) detector. A mobile phase of 0.1% trifluoroacetic acid in water at 2 ml/min was used with this column. Injection volume was 20 μl of sample. Chromatographic peaks were detected by their UV absorbance at 254 nm with a sensitivity of 0.1 AUFS unless otherwise noted.

Fly Factor Assay. An agar-base diet developed by Ignoffo and Gard (1970) was used for bioassay of β-exotoxin preparations

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against larvae of the house fly. Twenty-five third-instar larvae per test (in duplicate) were transferred to a petri dish containing 13.5 ml of diet which had been mixed with 1.5 ml of the sample. All β-exotoxin samples were heat-treated (80° C for 10 min) before assay. The plates were covered with filter paper and incubated at 30° C through the pupal stage until adult flies emerged in the control (approximately 10–12 days at 25° C and 60% relative humidity). Adult emergence as well as mortality during larval and pupal stages were recorded.

β-Exotoxin Standard. β-Exotoxin was supplied as Thuringiensin A (95% pure) from International Minerals and Chemical Corporation, Libertyville, Illinois, through the courtesy of T. R. Shieh. Additional samples of purified β-exotoxin were received from Sandoz, Inc., Homestead, Florida.

Results and Discussion

β-Exotoxin is an adenosine derivative linked through a glucose moiety to the 5′-position of phosphoallaric acid, with an approximate molecular weight of 800 (Farkas et al. 1969; Bond et al. 1969; Kalvoda et al. 1973). Its size and composition make it a likely candidate for separation by the techniques of HPLC. The method described by Oehler et al. (1982) initially appeared suitable for the detection of β-exotoxin when purified standards were employed. In our hands, the retention time (RT) of purified β-exotoxin was 6.97 min, approximately 3 min longer than reported by Oehler et al. (1982). Furthermore, 95% β-exotoxin routinely yielded a coincident peak at 6.0 min, apparently due to the existance in dry powders of the γ-lactone form of allaric acid (Kim and Huang 1970). The γ-lactone derivative was

converted to the free acid (Thuringiensin A) by alkaline treatment or by Dowex-1 (OH⁻) (Kim and Huang 1970). Although both derivatives possessed fly factor toxicity, the free acid form was about 8x more toxic to fly larvae. The elution pattern of Dowex-1 (formate⁻) treated standard yielded a single peak at 6.97 min (Fig. 1). The sharp peak at 1.88 RT resulted from formate in the sample buffer. The ultraviolet absorbance pattern of the eluate collected from peak 6.97 RT in 0.1% trifloroacetate revealed a strong maximum A_o at 254 nm and a minimum A_o at 230 nm. This material, when bioassayed as the 95% Thuringiensin A standard, was highly toxic toward flies.

Biological activity was measured using the fly factor assay. Emergence of adult flies after pupation is inhibited by exposure to β-exotoxin during the larval stage. Even among the survivors that emerge, badly deformed or missing body parts are often observed. The mortality to larvae and puparia, as well as the adult emergence is listed in Table 1. The standard β-exotoxin preparation was the same as that used for HPLC, and crude B. thuringiensis (B-4039) culture filtrate was diluted (1 : 1,000) to obtain an estimated toxicity. Mortality data were insufficient for probit analysis but adequate to establish a correlation between toxicity of the β-exotoxin preparations (both standard and culture filtrates) and potential detection via HPLC.

All B. thuringiensis strains do not produce β-exotoxin (Kim and Huang 1970). Those that do produce the toxin belong to serotypes 1, 8a/8b, 9, 10, and 11a/11b (deBarjac et al. 1966; Ohba et al. 1981).

Table 1. Mortality of the house fly (Musca domestica) to purified β-exotoxin and culture filtrates of Bacillus thuringiensis serotypes 1 and 3a

Treatment	No. of larvae	Mortality		Emergence	% Survival[a]
		Larvae	Puparia		
β-Exotoxin Std.					
5 µg/ml	100	1	99	0	0.0
1 µg/ml	50	2	34	14	35.9
B. t. var. thuringiensis					
Undiluted[b]	50	4	46	0	0.0
1 : 10	50	4	46	0	0.0
1 : 100	50	2	47	1	2.6
1 : 1,000	50	0	12	38	97.0
B. t. var. alesti					
Concentrate (2.5)	50	1	28	21	54.0
Undiluted	50	1	17	32	92.0
1 : 10	50	1	7	42	100.0

[a] Percent survival calculated on the basis of 78% emergence of 150 untreated control larvae
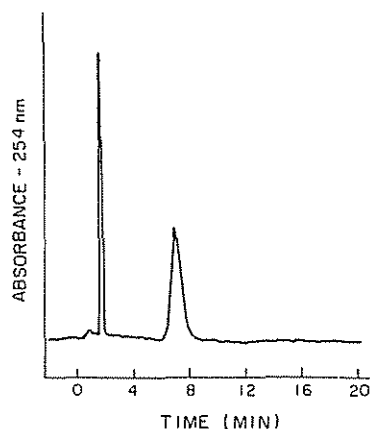[b] Culture filtrate dilution factor

Fig. 1. HPLC chromatogram of 95% pure Thuringiensin A after column chromatography through Dowex 1 (formate⁻) resin and elution with a 1–3 N formic acid gradient (15). Full scale: 0.05 AU

Different isolates of *B. thuringiensis* may or may not produce β-exotoxin depending upon the cultural conditions employed. Consequently, cultures of *B. thuringiensis* varieties *thuringiensis, alesti, kurstaki,* and *tolworthi* were grown in a yeast extract-glucose broth and in NSMP with supplemental sucrose. When examined by HPLC, no compounds co-eluted with the active standard peak (RT 6.97) in any of the culture filtrates from *B.* thuringiensis var. *thuringiensis* (Fig. 2). These filtrates did contain active fly factor activity, however, as seen in Table 1. Yet, filtrates of *B. thuringiensis* var. *alesti*, (a strain which fails to produce active fly factor toxicity) displayed a small peak coincident with the γ-lactone derivative of β-exotoxin (RT 6.0) (Fig. 2). Occasionally, certain strains yielded compounds which co-eluted with β-exotoxin, including *B. thuringiensis* var. *tolworthi* (serotype 9) and non β-exotoxin producers *B. sphaericus* and *B. subtilis.* Suspect peaks from these organisms were collected during elution, but none exhibited a maximum A_0 at 254 nm.

It is of interest to note that although Oehler et al. (1982) indicated the presence of β-exotoxin co-eluting material in culture filtrates of *B. thuringiensis* var. *darmstadiensis,* they provided no biological assay data to support their claim. In fact, Mohd-Salleh et al. (1980) found that *B. thuringiensis* var *darmstadiensis* failed to produce any β-exotoxin in a mineral casein medium similar to that used by Oehler et al. (1982). Clearly, the appearance of a peak which co-elutes with active β-exotoxin cannot be interpreted as direct evidence of the presence of β-exotoxin until more information is known. The existance in culture filtrates of contaminating compounds make interpretation of HPLC elution patterns difficult. A bioassay of the suspect peak must be performed to

establish fly toxicity before identification can be confirmed.

These findings limit the use of HPLC for detection and quantification of β-exotoxin. Additional analytical data must be gathered on the synthesis of β-exotoxin and similar molecules by classical negative fly factor *B. thuringiensis* serotypes before the potential usefulness of HPLC for β-exotoxin measurement can be determined.

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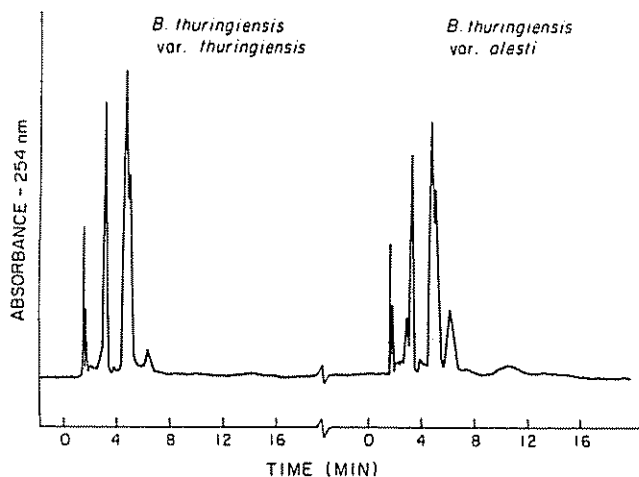

Fig. 2. HPLC chromatograms of crude culture filtrates of two varieties of *B. thuringiensis* grown on NSMP

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